Effects of Growth Regulators on In Vitro Plant Regeneration in Durum Wheat

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ABSTRACT

Work on improvement of durum wheat (*Triticum turgidum* L.) using tools of biotechnology is limited. Development of a reliable in vitro plant regeneration procedure for this important cereal is a prerequisite for its improvement by genetic transformation. Here, we report the effects of three growth regulators (GRs), 2,4-D (2,4-dichlorophenoxyacetic acid), picloram (4-amino-3,5,6-trichloropicolinic acid), and dicamba (3,6-dichloro-o-anisic acid), on callus induction and plant regeneration from scutellum cultures of four commercial durum cultivars: Ben, Maier, Munich, and Lebsock. Callus induction was obtained from isolated scutella cultured on modified Murashige and Skoog (MS) basal medium. After 4 wk of callus induction, all calli were plated on MS basal medium for regeneration. The regenerated plantlets were fertile, maintained the normal chromosome number ($2n = 4x = 28$) and structure as revealed by fluorescent genomic in situ hybridization (fl-GISH), and showed no apparent somaclonal variation. Genotype and callus induction medium played a dominant role in plantlet regeneration. Dicamba proved the best GR for inducing compact callus and also gave the highest proportion (0.16) of regenerated plants across the four cultivars. Overall, Maier gave the highest proportion (0.27) of plantlet regeneration when dicamba at 2.0 mg L^{-1} concentration was used for initial callus induction. These results will facilitate genetic transformation work with durum wheat.

Durum wheat ($2n = 4x = 28$; AABB) is an important cereal crop used for human consumption worldwide. Because of its high protein content and gluten strength, it is the wheat of choice for preparing pasta products. It is an important crop of the Northern Great Plains of the USA. Of the total durum wheat grown in the USA in the year 2000, 71.3% was in the state of North Dakota (USDA-NASS, 2001). Durum is also grown in several European countries including Italy, France, Turkey, Romania, and Ukraine, and in Canada.

Chromosome-mediated gene transfers, involving sexual hybridization coupled with manipulation of pairing among chromosomes of parental cultivars or species, have resulted in genetic improvement of both bread wheat (*Triticum aestivum* L.) and durum wheat (Jauhar, 1993; Friebe et al., 1996; Jauhar and Chibbar, 1999; Jauhar, 2003). However, this sexual technique of germplasm enhancement is time-consuming and has its own limitations (Jauhar, 2001; Repellin et al., 2001). In recent years, direct introduction of foreign DNA by modern techniques has shown considerable potential for genetic enrichment of cereal crops, including hexaploid wheat (Vasil et al., 1993; Weeks et al., 1993; Nehra et al., 1994; Blechl and Anderson, 1996; Clausen et al., 2000; Li et al., 2003). Extensive reviews on this subject are also available (Dahleen et al., 2001; Patnaik and Khurana, 2001; Rakszegi et al., 2001; Repellin et al., 2001; Janakiraman et al., 2002).

A reliable in vitro plant regeneration protocol is a prerequisite for the application of biotechnological methods in crop improvement. Although significant progress has been made in the transformation of cereals including bread wheat, similar research in durum wheat is still limited. A major obstacle to genetic transformation of durum was the lack of an efficient in vitro regeneration system. Bommineni and Jauhar (1996) standardized a regeneration protocol for four durum cultivars and subsequently, using this protocol, they produced transgenic durum wheat (Bommineni et al., 1997). Since this first report of genetic transformation of durum wheat there have been several reports on production of transgenic durum (He et al., 1999; Pellegrineschi et al., 2002). It would be advisable to use current commercial durum cultivars for genetic transformation to introduce new traits so that the transformants are directly useful. Hence, an efficient regeneration protocol applicable to current commercial cultivars is highly desirable. This report deals with the effects of three GRs, 2,4-D, picloram, and dicamba, on callus induction, callus regeneration capacity, and plant regeneration from scutellum cultures of four commercial durum cultivars: Ben, Maier, Munich, and Lebsock.

MATERIALS AND METHODS

Plant Materials: Preparing for Culture

Four agronomically important durum cultivars (Ben, Maier, Munich, and Lebsock) were selected to standardize the in vitro culture protocol. These cultivars were grown in the field under uniform conditions at the Casselton Seed Farm, North Dakota State University Experiment Station. Spikes from all four cultivars were collected approximately 2 wk post anthesis (summer season, 2002), labeled, and kept in a refrigerator at 4°C until the scutella were extracted for culture. Immature caryopses from spikes of all cultivars were removed at the same time for each replication. Thus, spikes of all cultivars in an experiment were in the refrigerator for the same amount of time. Immature caryopses were surface sterilized with 70% ethyl alcohol for 10 min, followed by 15% commercial bleach (active ingredient 5.25% sodium hypochlorite) with 0.2% polyoxyethylene sorbitan monolaurate for 30 min. Explants were then rinsed with sterile, distilled water three times (each wash for about 5-min duration) and used for culturing.

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Abbreviations: fl-GISH, fluorescent genomic in situ hybridization; GR, growth regulator; MS, Murashige and Skoog.

Callus Induction and Plant Regeneration

Immature embryos were excised from the caryopses under aseptic conditions. The scutella were isolated carefully by removing embryonic axes and any 20 scutella (derived and pooled from different spikes of each cultivar) were cultured per GR concentration in two Petri dishes, 10 in each. The scutella were placed (with cut surface of the scutellum in contact with the medium) in 15-mm high by 100-mm-diam. Petri dishes containing MS (Murashige and Skoog, 1962) medium supplemented with 30 g L^{-1} sucrose, 100 mg L^{-1} casein hydrolysate, and 100 mg L^{-1} myo-inositol. Four different concentrations, 0.5, 1.0, 2.0, and 2.5 mg L^{-1}, of each of the GRs, 2,4-D (2.26, 4.52, 9.05, and 11.31 μM), picloram (2.07, 4.14, 8.28, and 10.35 μM), and dicamba (2.26, 4.52, 9.05, and 11.31 μM), were used in different media. The medium was solidified with 0.8% purified agar (Sigma Chemical Co., St. Louis, MO) and autoclaved at 120°C for 20 min. Dicamba, being heat sensitive, was filter-sterilized and added to autoclaved medium. However, 2,4-D and picloram are not affected by heat and were therefore coautoclaved with the media (Sigma Biosciences, 1996). The cultures were incubated in the dark at 25 ± 2°C for 4 wk for callus induction.

After 4 wk, the callus was transferred to hormone-free MS medium. This regeneration medium was the same as the callus-induction medium but without hormones. The callus obtained from each explant was maintained separately. The cultures were placed in an incubation room at 25°C, illuminated with two warm white and two cool white, automatically timed fluorescent lights (3.1–5.5 μmol m^{-2} s^{-1}) with a 16-h photoperiod. Four weeks later, healthy plants with a well developed root and shoot were transferred to peat pellets and kept under the same growth-room conditions to harden before transplanting in a greenhouse. After 1 wk, the plants were transferred to 13-cm-diam. pots (filled with Sunshine Mix No. 1, Sun Gro Horticulture, Bellevue, WA) in a greenhouse. When established, the plantlets were transferred to bigger pots and grown to maturity.

Experimental Design

A replicated (four cultivars × three GRs × four concentrations) experiment was designed to study the effect of three GRs (2,4-D, picloram, and dicamba) on callus induction in four durum cultivars. Twenty scutella were cultured per GR concentration, with 10 explants per Petri dish. The experiment was repeated three times. Data were pooled from about 60 scutella per concentration of each GR. The number of scutella callusing (callus induction rate) was scored 3 wk after culture. The number of calli with somatic embryos (that were differentiating into green shoots) was scored 1 wk after transfer to regeneration medium. Three weeks later, the number of regenerated plantlets longer than 3.0 cm and with well-developed shoot and root system were scored before transfer to soil pellets.

Statistical Analyses

There were three data sets for which we performed three statistical analyses to determine (i) whether or not each explant produced calli; (ii) whether or not each explant with calli produced shoot buds, and (iii) how many plantlets each explant with shoot buds regenerated. The data on the number of explants callusing and the number of calli showing shoot buds (Cases i and ii) were analyzed as a 4 by 3 by 4 factorial design using a logistic regression model, where the response variable is presence or absence of some outcome event, and therefore considered binary. It must be emphasized that the traditional methods of linear regression and ANOVA are not applicable for analyzing these data because the assumptions of analyses were not met. This model (Hosmer and Lemeshow, 1989) posits a nonlinear model for the probability of the trait and can flexibly incorporate categorical or continuous predictors. The Hosmer and Lemeshow test was used to test the adequacy of the logistic regression model. Chi-square tests have been traditionally used to detect significant differences among the levels of each factor. In the present study, the logistic model posited the probability p_{ijk} of an explant showing callus (Case i) in terms of three predictors (covariates), which were all categorical: i = cultivar at Levels 1, 2, 3, and 4 (Ben, Lebsock, Maier, and Munich); j = GR at Levels 1, 2, and 3 (2,4-D, picloram, and dicamba); k = concentration of GRs at Levels 1, 2, 3, and 4 (0.5, 1.0, 2.0, and 2.5 mg L^{-1}). Theoretically, the logistic regression model fitted was as follows:

$$\ln[p_{ijk}/(1 - p_{ijk})] = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{i,j} + (\alpha\gamma)_{i,k} + (\beta\gamma)_{j,k} + (\alpha\beta\gamma)_{i,j,k},$$

where ln = natural logarithm; μ = general effect; α_i = effect of ith cultivar; β_j = effect of jth GR; γ_k = effect of kth concentration; $(\alpha\beta)_{i,j}$ = interaction between ith cultivar and jth GR; $(\alpha\gamma)_{i,k}$ = interaction between ith cultivar and kth concentration; $(\beta\gamma)_{j,k}$ = interaction between jth GR and kth concentration; and $(\alpha\beta\gamma)_{i,j,k}$ = interaction between ith cultivar, jth GR, and kth concentration.

For the analysis of the data on calli showing shoot buds (Case ii), the model posited probability p_{ijk} of calli showing shoot buds in terms of three predictors. The model was similar to the one presented above.

Data on the number of plantlets regenerated (Case iii) were analyzed with a Poisson Regression Model, where the response variable Y_{ijk} was a count. (Counts are traditionally modeled by a Poisson distribution.) In this case, the model posited the expected or average count EY_{ijk} of the number of plantlets obtainable and the offset variable was the number of explants cultured. This model had three categorical variables: i = cultivar, j = GR, and k = concentration, and was represented as follows:

$$\ln(EY_{ijk}) = \beta_0 + \beta_1\,(2,4\text{-D}) + \beta_2\,(\text{dicamba}) + \beta_3\,(\text{Ben}) + \beta_4\,(\text{Lebsock}) + \beta_5\,(\text{Maier}) + \beta_6\,(0.05) + \beta_7\,(1.0) + \beta_8\,(2.0) + \ln(\text{explants}).$$

SAS (SAS Institute, 2001) procedures were used for statistical analyses.

Cytological Studies

For cytological observations, root tips from 85 regenerated plants picked at random at the four-leaf stage were collected in chilled distilled water, kept at 4°C for 24 h, and then fixed in acetic alcohol (3:1, 95% ethanol to glacial acetic acid). The fixed root tips of 80 regenerants were squashed and stained with carbol fuchsin according to the method described by Jauhar et al. (1999). To discern the details of chromosome complement, somatic spreads of selected regenerants were studied using both conventional staining and fl-GISH techniques standardized earlier (Jauhar et al., 1999, 2000). Ten regenerants, selected at random, were studied by fl-GISH. Somatic chromosome spreads of durum regenerants were hybridized with *Triticum urartu* Tumanian ex Gandilyan genomic DNA [labeled with biotin-14-dATP (Gibco BRL, Gaithersburg, MD) 100 ng per slide], blocking the B genome with *Aegilops speltoides* Tausch genomic DNA (500 ng per slide). The chromosome preparations were counterstained with pro-

pidium iodide and the labeled DNA was detected with fluorescein isothiocyanate.

RESULTS

All four durum cultivars (Ben, Maier, Munich, and Lebsock) produced calli from isolated scutellum cultures. However, they differed in their abilities to produce calli on various media. The stages of callus and embryo formation and plant regeneration are represented in Fig. 1. Swelling of the explant was observed within 2 to 3 d after culture, and initiation of callus was apparent as a white translucent tissue on the surface of the scutellar region within 3 to 7 d, depending on the cultivar and medium. The appearance of dense, translucent tissue was an indication of the cell division activity, resulting in tissue clusters within 2 wk of incubation, as shown earlier by Bommineni and Jauhar (1996). Some of these tissue clusters gradually converted into compact white or yellow embryogenic calli (Fig. 1A). The percentage of explants callusing on average varied from 13 to 93%, with Lebsock showing the highest callus induction rate on 2.0 mg L^{-1} dicamba medium, and Munich showing the least on 2.5 mg L^{-1} picloram. Callus was white, friable/watery on the 2,4-D medium, white and friable on picloram medium, and highly compact/friable, slightly yellow on the dicamba medium.

Generally, the embryogenic calli differentiated into somatic embryos within 3 to 4 wk of culture on auxin-containing medium. After 4 wk of callus induction, the callus was carefully subdivided and transferred to fresh hormone-free MS medium. When exposed to light, the somatic embryos differentiated into green shoot buds (Fig. 1B). On picloram- and dicamba-containing media, shoot-like structures developed even during the dark induction period (Fig. 1C). The shoot buds grew into plantlets with well-developed root systems after 3 to 4 wk of culture on MS hormone-free medium (Fig. 1D).

The overall effects of the cultivar, GR, and its concentration on proportion of explants callusing, calli showing shoot buds, and plantlets regenerated were studied by taking one parameter and grouping the other two and are presented in the tables and Fig. 2. For callus induction, all the second-order interactions were significant and the relevant statistical analyses are presented in Tables 1 through 4. For calli showing shoot buds, no interactions were significant, and only the main effects were significant and the relevant statistical analyses are presented in Tables 5 through 8. Finally, for plantlet regeneration, only the main effects were significant, for which the relevant statistical analysis is given in Table 9. Other summary statistics are presented in Fig. 2.

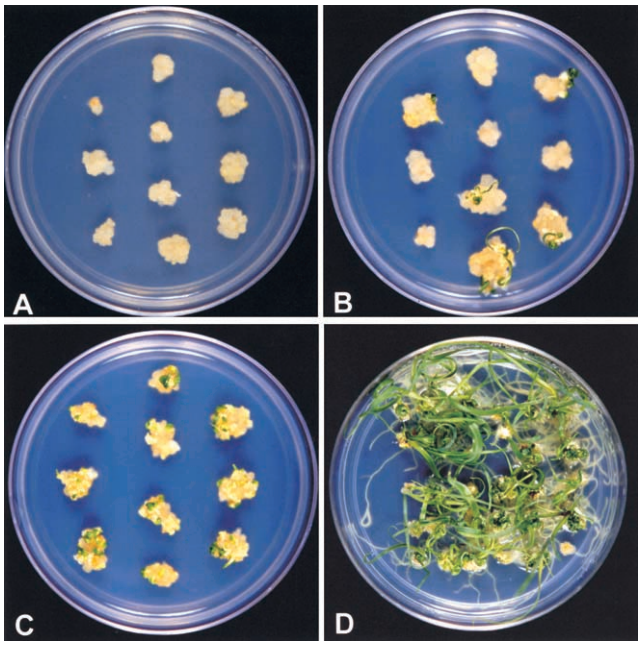

Fig. 1. Callus formation and plantlet induction in durum wheat cultivars. (A) Scutella showing calli from cultivar Lebsock, 3 wk after culture on modified Murashige and Skoog (MS) medium containing 2.0 mg L^{-1} dicamba. (B) Callus showing shoot buds of cultivar Maier, 4 wk after culture on medium containing 2.0 mg L^{-1} 2,4-D (green shoot-like structures developed after exposure to light), and (C) 2.0 mg L^{-1} dicamba (green shoots developed in the dark. (D) Plantlet regeneration from cultivar Maier on MS basal medium from callus initiated on 2.0 mg L^{-1} dicamba.

Callus Induction

Main Effects

If we focus on the main effects, the four cultivars showed significant differences in callus production (Table 1). Lebsock and Maier were clearly the two best

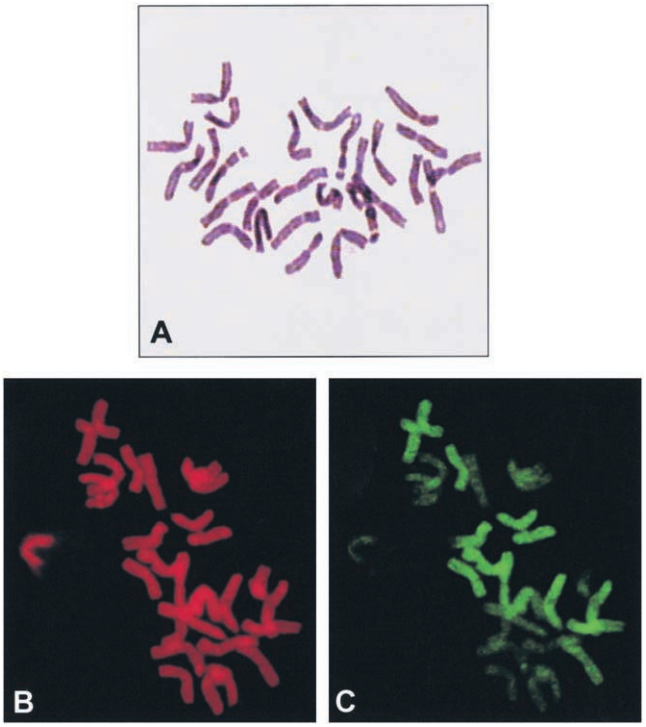

Fig. 3. Somatic metaphase chromosomes from root-tip cells and fluorescent genomic in situ hybridization of Maier regenerants from callus obtained on 2.0 mg L^{-1} dicamba. (A) 28 somatic chromosomes; (B) A 28-chromosome cell counterstained with propidium iodide (PI); and (C) Same cell as (B) hybridized with total genomic DNA of *Triticum urartu* and detected with fluorescein isothiocyanate (FITC). Brightly lit chromosomes belong to the A genome, while the faded chromosomes are from the B genome.

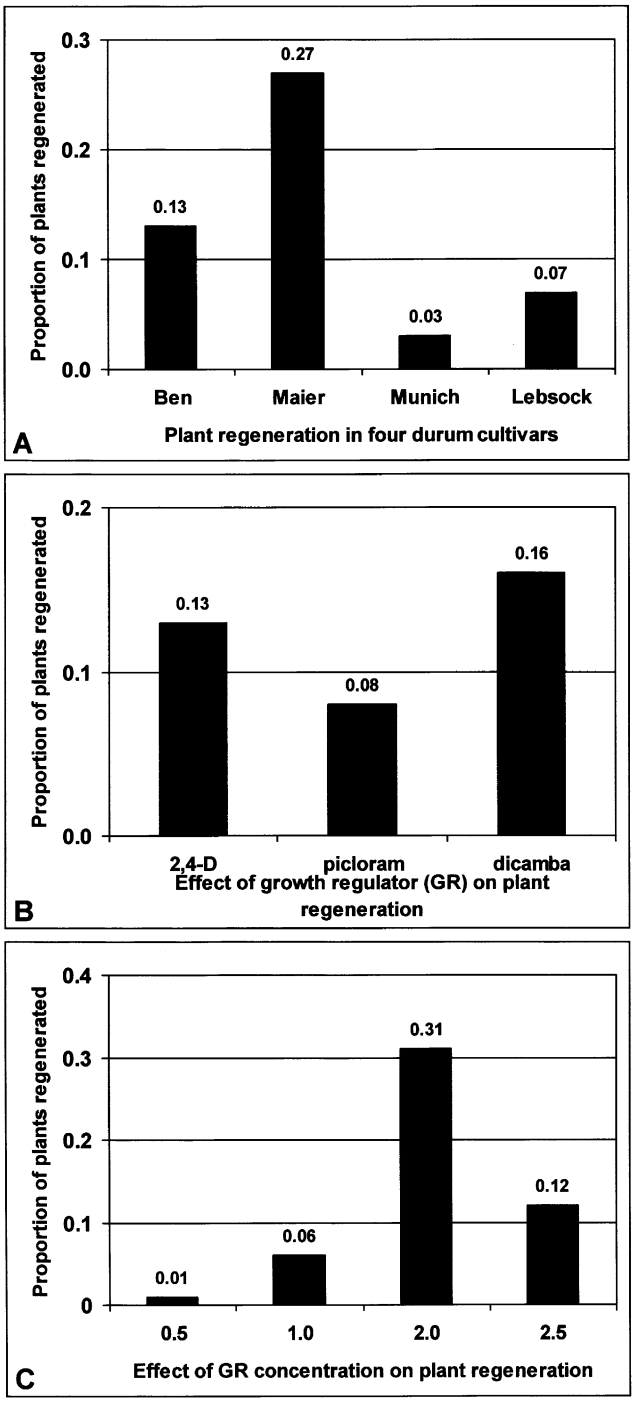

Fig. 2. Effects of (A) the cultivar, (B) growth regulator, and (C) the concentration of the growth regulator on proportion of plants regenerated.

producers of callus. Lebsock produced the highest number of calli (Table 2) and it was significantly different from Maier ($p = 0.006$). Among the three GRs, regardless of all other factors, dicamba proved to be the best for callus induction ($p < 0.0001$, Table 2). Of the four concentrations of GRs, 2.0 mg L^{-1} was found to be better than other concentrations ($p = 0.022$, Table 3).

When a logistic regression model with main effects and second-order interactions was applied to the data, the fit was good (Table 1, Hosmer and Lemeshow χ^2 test statistic value of 2.9496 at 8 df, $p = 0.9375$). However, third-order interactions were not significant and are therefore not shown in Table 1. The growth regulator and its concentration in the medium had a pronounced effect on callus production by the cultivars. Thus, all second-order interactions were also significant and were further analyzed to determine the best combination.

Table 1. Analysis of effects for callus induction using the logistic regression model.†

Effect	df	χ^2 value	p value
Cultivar	3	135.8034	<0.0001
Growth regulator (GR)	2	52.5776	<0.0001
Concentration	3	120.4631	<0.0001
Cultivar × GR	6	31.2348	<0.0001
Cultivar × concentration	9	21.6079	0.0102
GR × concentration	6	26.5882	0.0002

† Hosmer and Lemeshow (1989) χ^2 value test of goodness-of-fit of the model 2.9496 at 8 df, $p = 0.9375$.

Table 2. Percentages of callus induction for cultivar × growth regulator (GR) interaction.†

	Cultivar					
GR	Ben	Maier	Munich	Lebsock	Mean	SE‡
	%					
2,4-D	42.0 (224)§	60.8 (227)	49.3 (229)	63.3 (240)	54.0	0.04
Picloram	45.6 (226)	56.7 (238)	24.2 (240)	61.6 (232)	46.9	0.04
Dicamba	53.4 (234)	66.3 (240)	52.1 (240)	79.6 (220)	62.5	0.03
Mean	47.1	61.3	41.7	67.9		
SE‡	0.05	0.04	0.05	0.04		

† Chi-squared statistics for comparing 12 combinations of cultivar × GR interaction was 199.0525 at 11 df ($p < 0.0001$) with sample size 2790. Chi-squared statistics for comparing Lebsock × dicamba and Maier × dicamba was 10.2019 at 1 df ($p = 0.0014$) with sample size 460.
‡ SE is the standard error of the relevant mean percentage.
§ Number in parentheses represents the total number of explants used with a particular combination of GR and cultivar.

Table 3. Percentages of callus induction for growth regulator (GR) × concentration interaction.†

	Concentration, mg L^{-1}			
GR	0.5	1.0	2.0	2.5
	%			
2,4-D	42.9 (240)‡	46.1 (219)	63.0 (224)	64.1 (237)
Picloram	36.6 (232)	42.9 (238)	63.3 (226)	45.4 (240)
Dicamba	40.9 (230)	63.0 (235)	72.9 (240)	72.9 (229)
Mean	40.2	50.7	66.5	60.6
SE§	0.05	0.07	0.07	0.04

† Chi-squared statistics for comparing 12 combinations of GR × concentration interaction was 180.5799 at 11 df ($p < 0.0001$) with sample size 2790. Chi-squared statistics for comparing dicamba × 2.0 mg L^{-1}, 2.5 mg L^{-1} and 2,4-D × 2.5 mg L^{-1} was 5.7843 at 2 df ($p = 0.0555$) with sample size 706.
‡ Number in parentheses represents the total number of explants used with a particular combination of GR and concentration.
§ SE is the standard error of the relevant mean percentage.

Interactions

All 12 combinations of the cultivar × GR interactions were significantly different as shown in Table 2 (χ^2 value of 199.0525 at 11 df, $p < 0.0001$). The combinations Lebsock × dicamba, and Maier × dicamba showed the best response with probabilities of 79.6 and 66.3%, respectively (Table 2). When these two combinations were further compared, the combination Lebsock × dicamba

Table 4. Percentages of callus induction for cultivar × concentration.†

Cultivar	Concentration, mg L^{-1} 0.5	1.0	2.0	2.5
	%			
Ben	37.5 (176)‡	44.7 (170)	56.3 (160)	50.6 (178)
Maier	48.9 (178)	53.7 (175)	72.4 (172)	70.2 (178)
Munich	30.6 (180)	38.5 (169)	51.7 (180)	46.1 (180)
Lebsock	44.1 (168)	65.2 (178)	85.2 (176)	76.5 (170)

† Chi-squared statistics for comparing 16 combinations of cultivar × concentration interaction was 251.4756 at 15 df ($p < 0.0001$) with sample size 2790. Chi-squared statistics for comparing Lebsock × 2.0, 2.5 mg L^{-1} and Maier × 2.0 mg L^{-1} was 8.7735 at 2 df ($p = 0.0124$) with sample size 520.
‡ Number in parentheses represents the total number of explants used with a particular combination of cultivar and concentration.

Table 5. Analysis of effects for the number of calli with shoot buds using the logistic regression model.†

Effect	df	χ^2 value	p value
Cultivar	3	55.1856	<0.0001
Growth regulator	2	8.2430	0.0162
Concentration	3	179.5779	<0.0001

† Hosmer and Lemeshow (1989) χ^2 value test of goodness-of-fit of the model 16.2551 at 8 df, $p = 0.0389$.

Table 6. Percentages of calli with shoot buds for the effect of cultivar.†

	Cultivar Ben	Lebsock	Maier	Munich
	%			
Mean percentage	19.9	14.3	31.3	16.6
SE‡	0.11	0.07	0.11	0.13

† Chi-squared statistics for comparing among cultivars was 44.3786 at 3 df ($p < 0.0001$) with sample size 1520. Chi-squared statistics for comparing between Ben and Maier was 12.2859 at 1 df ($p = 0.0005$) with sample size 754.
‡ SE is the standard error of the relevant mean percentage.

proved to be the best statistically (χ^2 value of 10.2019 at 1 df, $p = 0.0014$).

The 12 combinations of the GR × concentration interactions (Table 3) were also significantly different for callus induction (χ^2 value of 180.5799 at 11 df, $p < 0.0001$). Dicamba at 2.0 mg L^{-1} and 2.5 mg L^{-1}, and 2,4-D at 2.5 mg L^{-1} were found to be more effective compared with others (Table 3). However, responses to dicamba (2.0, 2.5 mg L^{-1}) and 2,4-D (2.5 mg L^{-1}) were not statistically different from each other (χ^2 value of 5.7843 at 2 df, $p = 0.0555$).

All 16 combinations of the cultivar × concentration interactions were significantly different as shown in Table 4 (χ^2 value of 251.4756 at 15 df, $p < 0.0001$). However, for callus induction, Lebsock at 2.0 mg L^{-1} and 2.5 mg L^{-1} and Maier at 2.0 mg L^{-1} proved to be better combinations than others. When these combinations were compared among themselves, Lebsock at 2.0 mg L^{-1} concentration proved to be the best combination irrespective of the GR used (Table 4, χ^2 value of 8.7735 at 2 df, $p = 0.0124$).

Somatic Embryo Formation

The cultivar, GR, and concentration of GR had significant effects on number of calli showing shoot buds, an indication of somatic embryo formation. When a logistic regression model with main effects was applied to the data, the fit was good (Hosmer and Lemeshow χ^2 test statistic value of 16.2551 at 8 df, $p = 0.0389$). Thus, the main effects were significant (Table 5). However, for data on calli showing number of shoot buds, both second- and third-order interactions were not significant.

Table 7. Percentages of calli with shoot buds for the effect of growth regulator (GR).†

	GR 2,4-D	Picloram	Dicamba
	%		
Mean percentage	21.3	24.4	17.5
SE‡	0.08	0.09	0.06

† Chi-squared statistics for comparing among the growth regulators was 7.4432 at 2 df ($p < 0.0242$) with sample size 1520. Chi-squared statistics for between 2,4-D and picloram was 1.2301 at 1 df ($p = 0.2674$) with sample size 936.
‡ SE is the standard error of the relevant mean percentage.

Table 8. Percentages of calli with shoot buds for the effect of concentration of the growth regulator.†

	Concentration 0.5	1.0	2.0	2.5
	%			
Mean percentage	3.2	8.6	42.7	18.7
SE‡	0.06	0.08	0.11	0.09

† Chi-squared statistics for comparing among concentrations was 220.4641 at 3 df ($p < 0.0001$) with sample size 1520. Chi-squared statistics for comparing between 2.0 and 2.5 mg $L^{-1}$was 59.5678 at 1 df ($p < 0.0001$) with sample size 887.
‡ SE is the standard error of the relevant mean percentage.

Effect of Cultivar

All four cultivars were significantly different in their ability to produce calli with shoot buds, as shown in Table 6 (χ^2 value 44.3786 at 3 df, $p < 0.0001$). Maier was the best in producing calli with shoot buds (Table 6). Figure 1B and C shows calli with shoot buds of cultivar Maier induced on 2.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} dicamba, respectively.

Effect of Growth Regulator

As shown in Table 7, all the three GRs were also significantly different (χ^2 value of 7.4432 at 2 df, $p = 0.0242$). Of the three hormones, 2,4-D and picloram showed the best response (Table 7). On further comparison between the two, both 2,4-D and picloram were found to be equally effective in inducing calli with shoot buds (χ^2 value of 1.2301 at 1 df, $p = 0.2674$).

Effect of Concentration of Growth Regulator

The four concentrations were significantly different from each other (χ^2 value of 220.4641 at 3 df, $p < 0.0001$); 2.0 mg L^{-1} being the best in inducing calli with shoot buds (Table 8).

Plantlet Regeneration

Poisson regression analysis of the data on plantlet regeneration revealed significant differences among cul-

Table 9. Analysis of effects for the number of plantlets regenerated using the Poisson Regression Model.

Source	df	χ^2 value	*p* value
Cultivar	3	222.71	<0.0001
Growth regulator	2	34.62	<0.0001
Concentration	3	291.32	<0.0001

tivars, GRs, and their concentration for regeneration rates with no significant interaction among them (Table 9). For the purpose of comparison, we calculated the proportion of calli with shoot buds regenerating plantlets. Among the four cultivars, Maier showed higher proportion of plant regeneration compared with the other three cultivars in the order Maier (0.27) > Ben (0.13) > Lebsock (0.07) > Munich (0.03) (Fig. 2A). For plantlet regeneration, the effectiveness of GRs was dicamba (0.16) > 2,4-D (0.13) > picloram (0.08), irrespective of the cultivar used (Fig. 2B). The order of the most effective concentration for regeneration of plantlets was: 2.0 mg L^{-1} (0.31) > 2.5 mg L^{-1} (0.11) > 1.0 mg L^{-1} (0.06) > 0.5 mg L^{-1} (0.01) for the three GRs (Fig. 2C). Figure 1D, for example, shows regenerated plantlets of cultivar Maier from callus induced on 2.0 mg L^{-1} dicamba.

General Morphology of the Regenerants

The regenerants (10 per treatment) were studied morphologically with regard to general appearance, size, and leaf and spike characteristics. They all looked normal and similar to the maternal cultivar they were derived from. There was no evidence of apparent somaclonal variation.

Cytological Studies

All 80 regenerants studied showed 28 apparently normal somatic chromosomes (Fig. 3A). To determine whether the chromosome complement was intact, we did fl-GISH analysis on somatic chromosomes of 10 randomly picked regenerants obtained from callus on 2.0 mg L^{-1} dicamba. We found precisely 14 A- and 14 B-genome chromosomes (Fig. 3B and 3C), showing absence of aneuploidy or any chromosomal imbalance. There was no evidence of chromosomal aberrations.

DISCUSSION

Genetic transformation of a commercial cultivar would facilitate direct introduction of genes of interest into that cultivar and make it directly usable, thereby speeding up the breeding process. However, an efficient and reliable in vitro regeneration procedure is the first important step in any transformation protocol. In many cereal crops, including wheat and barley (*Hordeum vulgare* L.), immature scutellum has been the tissue of choice for in vitro plant regeneration and, hence, for genetic transformation (Barcelo and Lazzeri, 1995; Bommineni and Jauhar, 1996; Li et al., 2003). Auxins play an important role in somatic embryogenesis. The auxins commonly used are 2,4-D (Ahloowalia, 1982; Maddock et al., 1983), dicamba (Papenfuss and Carman, 1987), and picloram (Wernicke and Milkowitz, 1987), which vary in their efficacy according to plant species used. Therefore, we studied the effects of these GRs on in vitro plant regeneration in four commercial durum cultivars: Ben, Maier, Munich, and Lebsock.

The statistical analysis on callus production and plant regeneration showed significant differences among the four cultivars, the three GRs, and their concentrations. The study of the effects of genotype and culture medium, as well as any interactions between them using linear regression and ANOVA were considered inappropriate (Onay et al., 2000) because the fitted probabilities from such an analysis could be negative or >1. Moreover, the variances of their proportions were not constant, but depended on the corresponding probabilities. Therefore, use of logistic regression analysis in predicting the probabilities was considered more appropriate in analyzing data related to probabilities.

There were clear differences among the four durum cultivars in their capacity to produce callus or regenerate plants (Tables 1 and 9). Lebsock and Maier were the best producers of callus, and Maier produced the most regenerants. The presence of such genotypic differences is common in cereal crops. There are several reports of differences among cultivars of bread wheat (Caswell et al., 2000; Przetakiewicz et al., 2003) and durum wheat (Bommineni and Jauhar, 1996; Benkirane et al., 2000; Gonźalez et al., 2001).

We found dicamba to be more effective for callus induction and subsequent plant regeneration compared with the other two GRs, 2,4-D and picloram. These results are consistent with those of Mendoza and Kaeppler (2002), who studied effects of four GRs, 2,4-D, dicamba, picloram, and 2-MCPP [2-(2-methyl-4-chlorophenoxy) propionic acid] on callus induction and plant regeneration from mature embryos of wheat cv. Bobwhite. They found dicamba to result in a two-fold increase in the number of plants regenerated per embryo, and the amount of time required for plant regeneration was reduced by 3 to 4 wk. Previous reports on durum wheat tissue culture used 2,4-D at a concentration of 1.0–5.0 mg L^{-1} for callus induction (Borrelli et al., 1991; Bommineni and Jauhar, 1996).

In our study, the number of calli showing shoot buds was not significantly different on media containing 2.0 mg L^{-1} picloram or 2,4-D (Table 7). He and Lazzeri (2001) studied the effect of two auxins, 2,4-D and picloram, on scutellum culture response. They found that addition of auxin to media did not have a significant effect on embryogenesis, but it clearly affected regeneration, with cultures induced on picloram-containing media showing higher regeneration frequencies than those induced on 2,4-D. However, these workers did not compare the effect of dicamba in their studies. On the other hand, Hassan et al. (1999) found that picloram strongly inhibited somatic embryogenesis from mature embryo and hypocotyl cultures of oat. We also found that the proportion of plants regenerated from callus induced on picloram to be less than that on the dicamba medium (Fig. 2B).

Although Lebsock × dicamba and Maier × dicamba

were found to be a better choice for callus production, Maier gave the highest response with respect to plantlet regeneration when dicamba at 2.0 mg L^{-1} concentration was used for initial callus induction. Such an independent nature of callus induction rate and plantlet regeneration capacity support the suggestion of the presence of different genetic components controlling these traits as reported earlier in bread wheat (Chowdhury et al., 1991; Özgen et al., 1998) and durum wheat (Bohorova et al., 2001).

The use of medium with low auxin concentration or without auxin and/or addition of certain cytokinins seem to influence plantlet regeneration. In the present study, only MS medium without GRs was used for regeneration of plantlets. The percentage of plant regeneration varied from 0 to 31% with a mean value of 1.2 plantlets per scutellum, which seems to be lower than those reported by earlier authors. This might be because of the use of field-grown material, as the source of our explants, or the use of medium devoid of hormones for plantlet regeneration. Borrelli et al. (1991) reported regeneration frequencies varying from 0 to 7.2% in durum wheat, and Bommineni and Jauhar (1996) showed best values of 62 and 100% scutella-regenerating plants for four durum cultivars, with an average of 16 plantlets per scutellum cultured on RM2 (Regeneration Medium 2) containing 1.0 mg L^{-1} each of BA (6-benzylaminopurine) and IAA (Indole-3-acetic acid). He and Lazzeri (2001) reported a higher number of plantlet regeneration (34 plantlets per scutellum) with regeneration frequencies of 97 to 100% in durum wheat after one or two passages on regeneration medium containing zeatin [*N*6-(4-hydroxyisopentenyl)adenine], whereas Bohorova et al. (2001) reported regeneration values ranging from 0 to 100% with five to 20 plantlets per embryo cultured on MS medium supplemented with 0.5 mg L^{-1} IAA and 1.0 mg L^{-1} BA.

Although numerous workers have successfully used 2,4-D for callus induction, this auxin at higher concentrations is reported to increase chromosomal instability, leading to somaclonal variation (Karp, 1994). Therefore, other strong auxins such as dicamba and picloram have been used as alternatives (Pedrosa and Vasil, 1996). Dicamba was reported to equal or surpass 2,4-D in inducing shoot formation in maize (*Zea mays* L.; Carvalho et al., 1997), barley (Castillo et al., 1998; Trifonova et al., 2001), triticale (×*Triticosecale* spp.; Immonen, 1996; Ainsley and Aryan, 1998), and wheat (Hunsinger and Schauz, 1987; Papenfuss and Carman, 1987; Bahieldin et al., 2000; Mendoza and Kaeppler, 2002).

Tissue culture-induced variations in the regenerated plants of wheat have been explained by numerical or structural changes in chromosomes (Ahloowalia, 1982). Using doubled haploids of wheat, Marburger and Jauhar (1989) indicated the chromosomal basis of somaclonal and gametoclonal variations induced in tissue cultures. In the present study, the regenerated plantlets grew to maturity in greenhouse conditions without apparent morphological changes and showed the expected chromosome number of $2n = 4x = 28$ (Fig. 3A). Fluorescent GISH analysis of the chromosome complement showed 14 A- and 14 B-genome chromosomes (Fig. 3B and 3C) with no evidence of any chromosomal imbalance or abnormality.

CONCLUSIONS

We studied the effects of three GRs on callus induction and subsequent plant regeneration in four current commercial durum wheat cultivars. Overall, the results revealed dicamba as the most suitable auxin for callus formation and subsequent plantlet regeneration across all four cultivars. Maier had the highest percentage of scutellum-regenerating plants, and the highest number of plantlets regenerated per scutellum with dicamba at 2.0 mg L^{-1} concentration and thus is a choice cultivar for use in genetic transformation research.

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